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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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DEPARTMENT OF [illegible]

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NUCLEOSIDE TRANSPORT IN HUMAN ERYTHROCYTES

by

JANET MARY OLIVER

A THESIS
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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "NUCLEOSIDE TRANSPORT IN HUMAN ERYTHROCYTES", submitted by Janet Oliver in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The transport of purine and pyrimidine nucleosides, and the inhibition of this process by a 6-alkylmercaptopurine ribonucleoside, have been examined in human erythrocytes. Rates of nucleoside transport have been measured by an assay technique in which incubation mixtures were centrifuged through dibutyl phthalate, in order to achieve rapid separation of cells from medium. Interpretation of the results has been simplified by use of a non-metabolised nucleoside, uridine, as the permeant in investigations of the mechanism of transport.

It has been demonstrated that uridine transport is not accumulative and is independent of energy metabolism, indicating that the movement of nucleosides in erythrocytes is not mediated by an active transport system. Passive diffusion of nucleosides has also been excluded since uridine uptake has been shown to be a saturable and temperature-dependent process, and to be competitively inhibited by addition of a second nucleoside.

These properties suggest that a facilitated diffusion system is involved in nucleoside transport; this possibility has been confirmed by demonstration of the phenomenon of counterflow with uridine and inosine. Additional counterflow studies have shown that the facilitated diffusion system has a broad specificity for nucleosides, and that it is distinct from the mediated transport systems for purine and pyrimidine bases and for sugars in human erythrocytes.

It has been found that 2-amino-6-(p-nitrobenzylmercapto) purine ribonucleoside reacts competitively with the transport system to produce an apparently irreversible inhibition of nucleoside influx and efflux in intact erythrocytes and in erythrocyte 'ghosts'. This compound did not affect the mediated transport of bases or sugars, indicating that it may bind specifically with the membrane 'carrier' involved in nucleoside transport.

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LIST OF ABBREVIATIONS

EDTA	ethylenediaminetetraacetic acid
Tris	tris(hydroxymethyl)aminomethane
TES	*N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid
BzTG	2-amino-6-(benzylmercapto)purine
BzTGR	2-amino-6-(benzylmercapto)purine ribonucleoside
NBzTGR	2-amino-6-(*p*-nitrobenzylmercapto)purine ribonucleoside
Formycin B	3(β-D-ribofuranosyl)pyrazolo[4,3-*d*]-6(H)-7-pyrimidone
DBP	dibutyl phthalate (*n*-butylphthalate)
AICAR	5-amino-4-imidazolecarboxamide ribonucleoside

INTRODUCTION

I. Transport across Biological Membranes

The composition of the cytoplasm of living cells is different from that of the surrounding medium, not only with respect to large molecules, such as proteins and nucleic acids, but also with respect to many small molecules and ions. From this, it has been postulated that the interchange of substances across the limiting membrane of cells is strictly regulated; studies of the movement of many different molecules across biological membranes have supported this possibility. The processes which are most commonly involved in permeation may be divided into three broad classes; namely, pinocytosis, passive diffusion and mediated transport (1-4).

(i) Pinocytosis

In certain cell types, particularly amoeboid and epithelial cells, pinocytosis (and the related process of phagocytosis) may be involved in both the entrance and exit of some molecules. Material to be brought into the cell by this process is engulfed by the membrane to form an intracellular vesicle, while material to be extruded is enclosed in a vesicle which fuses with the cell membrane and discharges its contents into the extracellular medium. It has been shown by electron microscopy that large molecules, such as proteins and vitamins, may enter the epithelial cells of intestine and kidney by pinocytosis, and in amoebae and leucocytes, this process is readily observed by light microscopy. However,

the speed and selectivity of most transport processes suggests that pinocytosis may be an auxilliary mechanism rather than an alternative to membrane transport.

(ii) <u>Passive Diffusion</u>

In passive diffusion, permeants usually move in or out of cells along their individual concentration gradients across the membrane. This type of transport is limited by the ability of the permeant to diffuse through the cell membrane. Since the rate of uptake of many non-electrolytes is directly related to their oil-water partition coefficients, it has been postulated that lipophilic compounds diffuse into the cell by dissolving in the lipid of the cell membrane. A number of small hydrophilic molecules, whose movement would be greatly restricted by a lipid barrier, also move passively; permeation of these compounds is thought to involve diffusion along aqueous pores or channels in the membrane. Hydrophilic compounds may also permeate by 'solvent drag' in the flow of water through these pores. Selective diffusion of ions has been explained by postulating that the pores bear charged groups, so that ions of one charge are accepted, while those of the opposite charge are rejected.

(iii) <u>Mediated Transport Systems</u>

Mediated transport systems may be divided into three broad categories on the basis of the driving force involved in permeation. These are the facilitated diffusion systems, the primary active transport systems and the secondary active transport systems.

The driving force in facilitated diffusion is simply the concentration gradient of the permeant across the membrane. Hence, transport is independent of energy metabolism and accumulative uptake does not occur.

Both types of active transport involve the expenditure of metabolic energy and allow accumulation of substrates. The movement of permeants by primary active transport is coupled directly to the consumption of metabolic energy, generally in the form of ATP; a number of carbohydrates are concentrated in bacteria by this type of system (1). In secondary active transport systems, the uptake of a substrate against its concentration gradient is driven by the outward flow of a second permeant which has previously been accumulated in the cell. For example, the concentrative transport of glucose into the epithelial cells of the intestinal mucosa is thought to be driven by a coupled efflux of sodium ions effected by the sodium pump of these cells (5).

In all mediated transport systems, a component of the membrane facilitates the movement of substrates. This component is represented in 'fixed site' models by a number of separate and specific pores which allow the exit or entry of permeants. It is represented in 'carrier' models as a membrane-bound molecule, probably a protein, which is able to complex with substrate at one face of the membrane and release it at the other; the same component mediates both influx and efflux. Kinetic analyses of the sugar transport system of erythrocytes support the 'carrier' hypothesis rather than the 'fixed site' models (1).

As yet, the mechanism of 'carrier'-mediated transport is not fully understood. The simplest hypothesis proposes that the 'carrier'-substrate complex moves physically across the membrane, but Vidaver (6) has presented a model, based on the allosteric enzyme model (7), which also accounts for the kinetic characteristics of sugar transport into erythrocytes. He has postulated that the 'carrier' exists in two forms, R and T, and that each form can bind substrates only at one surface of the cell membrane. Permeants move in or out of the cell as a result of the interconversion of the two forms. Critical evaluation of these and other models will not be possible until kinetic studies of substrate binding and associated changes in conformation of the 'carrier' can be carried out with isolated transport systems.

II. Facilitated Diffusion

This term was introduced in 1954 by Danielli (8) to describe a number of transport systems in which movement occurred strictly along the concentration gradient of substrate across the membrane, but was more rapid than would be predicted from the lipid solubility, molecular size and charge of the permeants. Facilitated diffusion has since been demonstrated in a wide range of cell types and for a number of metabolites, principally sugars, amino acids and ions (1).

The kinetic properties of facilitated diffusion systems have been determined principally from studies on the transport of sugars and glycerol into human erythrocytes (1,9-11) and of amino acids into Ehrlich ascites tumor cells (12). Several characteristics which identify this transport mechanism become apparent when these properties are compared with the properties of the other types of membrane transport.

If accumulative uptake of a substance is observed, an active transport system must be operating. However, the demonstration of an equilibrating flow of permeant across a membrane is consistent with either facilitated or passive diffusion; studies which may decide which transport mechanism is involved are suggested by the flux equations describing these alternative processes.

It has been found that the unidirectional flux of a substrate of a facilitated diffusion system is described by the equation:

$$J = \frac{S\ V_{max}}{K_m + S} \qquad (i)$$

in which J is the unidirectional flux of permeant, S is the concentration of permeant at the side of the membrane from which flow is occurring, V_{max} is the rate of transport when all of the available 'carrier' is saturated with substrate, and K_m may express some type of 'affinity' of the substrate for the 'carrier' system. This equation is directly analogous to the Michaelis-Menten equation for the initial velocity of an enzymic reaction; it predicts that, with increasing substrate concentrations, the unidirectional flux will approach the saturation value, V_{max}. By extending further the analogy to enzyme kinetics, the addition of a second substrate of the 'carrier' system, with a dissociation constant, K_i, should lead to competitive inhibition of the movement of the first permeant; in this case, the term K_m in equation (i) becomes $(1 + [I]/K_i)\ K_m$, where [I] is the concentration of the inhibitor.

In contrast, unidirectional flux of a permeant moving by simple diffusion is described by Fick's law, which is stated simply as:

$$J = PAS \qquad (ii)$$

where P is a permeability constant, A is the area of the membrane, and S is the concentration of the permeant. Neither saturation of the transport process, nor competition between permeants, would be predicted for substances which enter cells by this mechanism.

It should be emphasized that an apparent absence of saturation kinetics does not necessarily prove that a permeant is not

transported by a 'carrier'-mediated system. For example, sorbose is a substrate of the erythrocyte sugar transport system, but the K_m is so high (approximately 500 mM) that uptake of sorbose shows non-saturating kinetics even at high concentrations (1). Under such circumstances, transport by facilitated diffusion may be recognised by the temperature dependence of uptake; in simple diffusion the Q_{10} value is rarely greater than 1.5, while 'carrier'-mediated transport usually has a Q_{10} of between 2 and 3 (13). An examination of the stereochemical specificity of transport may also distinguish between simple and facilitated diffusion, since in general, optical isomers of the same permeant move at similar rates by passive diffusion, but at different rates by facilitated diffusion. This stereospecificity is well illustrated in the sugar transport system of the human erythrocyte, where the K_m for the facilitated diffusion of D-glucose is 0.006 M and the K_m for L-glucose is greater than 3 M (14).

Demonstration of the phenomenon of counterflow (also known as counterflux, counter-transport, the trans effect and exchange diffusion) also indicates that a pair of compounds is transported by facilitated diffusion. This effect was first demonstrated by Rosenberg and Wilbrandt (15), who found that addition of mannose to a suspension of erythrocytes which had been equilibrated with glucose resulted in a transient flow of glucose out of the cells against its concentration gradient. Since both sugars use the same facilitated diffusion system, it was suggested that the 'uphill' efflux of glucose was a direct result of competition between

these compounds for transport. Similar effects have since been demonstrated with a number of pairs of permeants (1).

Counterflow may be explained in terms of the following model (1). If a suspension of cells is equilibrated with a substrate, A, then molecules of A will exchange freely across the membrane, but the resulting net flux (which is the difference between the inward and outward fluxes) will be negligible. The addition to the medium of B, a compound which shares the same transport system, will result in a competition between A and B for entry, and this competition will reduce the influx of A without affecting its efflux. As a result, more A will be leaving the cells than is entering and there will be a net outward transport of A, although there was initially no concentration gradient of A in this direction. This 'uphill' transport will persist as long as the external concentration of B is sufficient to suppress the influx of A and as long as the internal concentration of B is too low to inhibit the exit of A.

A second type of counterflow effect has been demonstrated in studies on the active transport of glycine into Ehrlich ascites tumor cells (16) and on the facilitated diffusion systems which move sugars and amino acids into erythrocytes (17-19). It was observed that when cells containing a substrate, A, were exposed to a large volume of medium lacking related permeants, a slow efflux of A occurred. However, if the medium contained a second substrate, B, which shared the same transport system, the rate of exit of A was greatly accelerated. This could not be

explained in terms of a decrease in the influx of A, since the medium contained a negligible amount of this substrate, and so it was suggested that the 'carrier' is a mobile component which moves from the outside of the membrane to the inside more rapidly when complexed with B than when it is free. The net result is a stimulation of the unidirectional flux of A by the second solute, B.

Investigation of the transport of amino acids (19) and of sugars (1) into human erythrocytes has shown that with different substrates of the same transport system, one may stimulate counterflux more than another. To explain this observation, it has been proposed that either the rate of movement across the membrane of the 'carrier'-substrate complex, or the rates of formation and dissociation of this complex, may be characteristic of the particular permeant associated with the transport system.

Production of counterflow is particularly useful in examining the substrate specificity of a facilitated diffusion system. For example, Winter and Christensen (19) found that the efflux of leucine from erythrocytes was accelerated by addition to the external medium of valine, methionine or phenylalanine, but was unaffected by the presence of threonine, alanine, glycine or tryptophan. They concluded that the latter amino acids do not share the leucine 'carrier' system.

III. The Transport of Purine and Pyrimidine Derivatives

A number of studies have been carried out on the transport of purine and pyrimidine compounds in various tissues. The results of these investigations are reviewed below.

(i) Bases

Lassen (13) and Lassen and Overgaard-Hansen (20-22) found that the uptake of uric acid into human erythrocytes was temperature-dependent (with a Q_{10} of 2.2), saturable and non-concentrative. Hypoxanthine and a number of purine and 8-azapurine derivatives competitively inhibited the influx of uric acid, implying that a facilitated diffusion system was involved in the transport of these bases. However, counterflow of uric acid was not induced by hypoxanthine, and it was observed, rather, that extracellular hypoxanthine caused a partial inhibition of uric acid efflux. These results are unexpected in a facilitated diffusion system, but may possibly have been due to a rapid inhibition of uric acid efflux, resulting from the high affinity of hypoxanthine for the transport system (K_m for uric acid was 3 m$\underline{M}$, K_i for hypoxanthine was 0.1 m$\underline{M}$). Lassen (23) has presented evidence that hypoxanthine transport may be mediated by a 'carrier' system at low substrate concentrations, and that passive diffusion may also occur at high concentrations of hypoxanthine.

Jacquez (24) showed that uracil, thymine and 5-fluorouracil equilibrate very rapidly between the cellular and extracellular water of a suspension of Ehrlich ascites tumor

cells. He concluded that these compounds enter the cell by simple diffusion; however, the temperature-dependence of base transport observed in these experiments does not appear consistent with this proposal.

Other studies have shown that human erythrocytes are highly permeable to hypoxanthine and adenine (25); and that orotic acid and 5-fluoroorotic acid enter ascites cells less rapidly than 5-fluorouracil and uracil (26,27).

Schanker and coworkers (28-30) have examined the transport of pyrimidines across the intestinal epithelium of rat, hamster and frog. Movement from the mucosal side to the serosal side of everted intestinal sacs was shown to involve a specific, saturable mechanism at low substrate concentrations and a passive, non-saturable component which was evident at high substrate concentrations. The saturable component appeared to be an active process, since accumulative transport of non-metabolised substrates into these sacs was demonstrated; this accumulation was depressed by anoxia and several inhibitors of energy metabolism. A number of purines and pyrimidines competitively inhibited the uptake of uracil and thymine, suggesting that a common transport system was responsible for the movement of these bases across the intestinal wall.

Czaky (31) found that omission of sodium or the presence in the medium of digitalis, a cardiac glycoside which specifically inhibits ion transport, blocked the active transport of uracil across intestinal epithelium. This indicated pyrimidine base transport may be mediated by a

secondary active transport system similar to that involved in the accumulation of glucose in the cells of the intestinal mucosa (Introduction I, (iii)).

Guthrie and Lu (32) and Demain (33) showed an inhibition of growth of purine-requiring strains of Bacillus subtilis after the addition of certain bases; for example, the growth of a guanine-less mutant was inhibited by adenine and hypoxanthine, but not by xanthine. Since the ribonucleosides of the inhibitory bases did not affect growth, these authors suggested that the inhibition was due to competition for transport between essential bases and added bases which shared the same 'carrier' system.

Roush et al. (34) found that an energy-dependent transport system in the yeast Candida utilis allows an accumulation of a number of purines, including isoguanine, a base which is not metabolised in these cells. Concentrative uptake of adenine has also been demonstrated in brewers yeast (35); a respiratory-deficient mutant was unable to accumulate the purine and this supports the evidence that base transport is dependent upon energy metabolism.

(ii) Nucleosides

Some evidence is available which suggests that a mediated process may be involved in the movement of nucleosides. For example, McLellan and Lionetti (36) and Whittam (25) observed a rapid uptake of inosine and adenosine into erythrocytes, and Heidelberger et al. (26) found that 5-fluorouridine readily enters Ehrlich ascites cells. It has also been shown that nucleotides are dephosphorylated as they cross the intestinal epithelium of hamster and rat (37),

or enter rat liver slices and Ehrlich ascites tumor cells (38). In this laboratory, Paterson and Simpson (39) have observed that erythrocytes from various animals transport nucleosides at different rates; these differences suggest that a process other than simple diffusion may be involved in nucleoside movement.

Paterson and Simpson have obtained evidence to support this possibility in studies with a group of inhibitors of synthetic, exchange and cleavage reactions involving nucleosides in intact cells of the Ehrlich ascites carcinoma and in intact erythrocytes of the mouse and human (40-42). The most effective inhibitors were 6-alkylmercapto derivatives of purine ribonucleosides and 2-aminopurine ribonucleosides; a large variety of substituents on the sulphur atom, ranging from methyl to p-nitrobenzyl, imparted inhibitory properties. Since none of these compounds affected nucleoside metabolism by broken cell preparations, it was suggested that they interfere with the entry of nucleosides into the cell, rather than with their subsequent metabolism. In studies on the guanosine-supported synthesis of inosine, a competitive relationship was established between guanosine and NBzTGR[1]. No competition was observed between hypoxanthine and the inhibitor (43), indicating that the nucleoside analogue may affect a transport system which is specific for nucleosides.

Jacquez (44) has examined the transport and phosphorolysis of pyrimidine nucleosides in Ehrlich ascites tumor

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[1] NBzTGR, 2-amino-6-(p-nitrobenzylmercapto)purine ribonucleoside.

cells. In this study, the initial rates of uptake were not measured and the results were complicated by intracellular metabolism of the substrates. However, it was shown that uptake was not concentrative and that the velocity of uptake ceased to be proportional to substrate concentration at high levels of permeants; from these observations, it was proposed that nucleosides enter Ehrlich cells by facilitated diffusion. Jacquez suggested that nucleosides may share the sugar transport system since phlorizin, glucose, galactose and 3-O-methyl-glucose all caused a slight inhibition of uridine influx.

The transport of cytosine arabinoside and deoxycytidine in cells of the L1210/CA murine leukemia, which are unable to metabolise either nucleoside, has been studied by Kessel and Shurin (45). The assay used did not measure the initial rates of influx or efflux, but it was found that uptake was saturable, non-accumulative and unaffected by inhibitors of energy metabolism. The presence of other nucleosides decreased the amount of cytosine arabinoside or deoxycytidine which entered or left the cells, but free bases, arabinose or cytidylic acid had no effect on nucleoside transport. These data support the suggestion of Jacquez that nucleosides may enter tumor cells by a common facilitated diffusion system. Kessel and Shurin observed an apparently concentrative uptake of nucleosides after addition of uranyl nitrate to the medium. From this, it was postulated that uranyl ions preferentially inhibit the exit of nucleosides, but not their entry.

Several reports on the uptake of nucleosides into sea urchin eggs (46), chick embryo cultures (47,48) and human

lymphocytes (49) have suggested that the transport of nucleosides may be linked with their phosphorylation. This possibility has been examined in cultured chick fibroblasts by Scholtissek (50). He found that persantin[2] was a competitive inhibitor of the transport and subsequent metabolism of nucleosides in these cells, and that removal of the inhibition could be effected only by the addition of a second nucleoside sharing the kinase responsible for the phosphorylation of the first permeant. From these data, he proposed that kinases may be involved in transport. His observation that persantin did not affect the nucleoside kinase activities of cell-free extracts of fibroblasts, led to a model in which uptake involved a nucleoside-kinase complex; persantin may interfere in some way with the nucleosides in this complex. The conclusions drawn by Scholtissek are difficult to assess, since the results reflect both the transport of substrates and their subsequent metabolism during the long incubation periods employed.

Considerably more detailed studies have been carried out by Koch and coworkers (51,52) on the transport of nucleosides into *Escherichia coli*. They found that influx was saturable and temperature dependent, that adenosine, cytidine and uridine competed with each other for entry, and that a number of nucleosides accelerated the efflux of inosine and uridine. These observations indicated that a common 'carrier' system was probably involved in nucleoside movement. It was shown that uptake could be coupled to energy metabolism to

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[2] Persantin, 2,6-*bis*(diethanolamino)-4,8-dipiperidinopyrimido(5,4-*d*)pyrimidine.

allow accumulation of substrates. However, in the presence of metabolic inhibitors, nucleosides were still able to equilibrate between the medium and the cytoplasm, indicating that the concentrating ability was probably not an integral part of the transport system. They also found that adenosine uptake was inactivated in cells which had been heated briefly, but cytidine and uridine transport were not affected; further, they observed that thiol reagents could prevent uridine and adenosine uptake without affecting the movement of cytidine. From these data, it was suggested that transport by the 'carrier' might involve prior reaction with a nucleoside-specific 'permease'. There are a number of precedents for this type of two-element transport system (53).

MATERIALS AND METHODS

I. Materials

Human erythrocytes were obtained from blood which had been stored for 21 to 25 days in acid-citrate-dextrose (ACD) medium; this was provided by the Blood Bank of the University of Alberta Hospital, through the courtesy of Dr. D. I. Buchanan. The Division of Hematology, Department of Medicine, University of Alberta Hospital, provided samples of venous blood which had been collected throughout the hospital for routine laboratory tests 12 to 16 hours previously in EDTA[3] anticoagulant; 2 to 3 ml portions from approximately 50 samples of this blood were pooled and used to prepare erythrocyte 'ghosts'.

L-leucine-^{14}C and inulin-carboxyl-^{14}C were purchased from the New England Nuclear Corporation; uracil-2-^{14}C was manufactured by the Volk Radiochemical Company; and all other radioactive compounds were obtained from Schwarz Bioresearch Inc.

Formycin B[4] was generously supplied by Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo; Dr. R. K. Robins, University of Utah, Salt Lake City, provided NBzTGR; and BzTG[5] and BzTGR[6] were supplied by the Cancer Chemotherapy National Service Centre, Bethesda, Maryland.

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[3] EDTA, ethylenediaminetetraacetic acid.

[4] Formycin B, 3(β-D-ribofuranosyl)pyrazolo-(4,3-d)-6(H)-pyrimidone.

[5] BzTG, 2-amino-6-(benzylmercapto)purine.

[6] BzTGR, 2-amino-6-(benzylmercapto)purine ribonucleoside.

Uridine, cytidine and the corresponding bases were obtained from the Pabst Laboratories; other nucleosides and bases were purchased from the California Corporation for Biochemical Research. 2-Deoxy-D-glucose and TES[7] were purchased from the Sigma Chemical Company; and UV-test kits for the assay of ATP from C. F. Boehringer and Soehne.

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[7] TES; N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid.

II. Nucleoside Metabolism

The ability of human erythrocytes to metabolise nucleosides was examined by methods which have been described previously (45). The cells were washed twice with 4 volumes of Krebs-Ringer phosphate medium (54) at room temperature, and were incubated in the same medium in a shaking water bath for 15 minutes at 37°. Duplicate assay mixtures contained 100 mg of washed cells (wet weight) in a final volume of 1.0 ml, and radioactive substrates were added to a concentration of 1.0 m*M*. After incubation, the cells were removed by centrifugation, the medium was heated for 2 minutes in a boiling water bath, and measured portions of medium were chromatographed on paper with appropriate nucleosides and bases as 'carriers'. The chromatograms were developed in *n*-butanol-acetic acid-water (5:3:2, v/v) to separate inosine and hypoxanthine, and in isobutyric acid-water-28% NH_4OH-0.1 *M* EDTA (100: 55.8: 4.2: 1.6, v/v) to separate uracil and uridine. 'Carrier' areas were cut from the chromatograms and counted directly in a toluene-fluor solution (55) in a liquid scintillation counter.

III. Transport Studies

(i) General

The buffered medium (medium A) which was used throughout the transport studies consisted of 140 m$\underline{M}$ NaCl, 18 m$\underline{M}$ TES (56) pH 7.4, 1.4 m$\underline{M}$ $MgSO_4$, 5 m$\underline{M}$ glucose. In all experiments, ACD-stored blood was centrifuged, the supernatant and buffy coat were removed, and the cells were washed twice at room temperature in 3 volumes of medium A. The subsequent steps were as described in the individual experiments. Centrifugations were for 4 minutes at approximately 3000 rpm in a bench centrifuge, unless otherwise noted.

The acid-soluble constituents of cells were separated from the acid-insoluble components by extracting erythrocytes twice with approximately 2 volumes of cold 4% perchloric acid, followed by neutralization of the extract with 3$\underline{N}$ KOH containing 0.1$\underline{N}$ Tris[8]. The insoluble $KClO_4$ was removed by centrifugation in the cold.

Measured volumes of packed cells were transferred with 1 and 5 ml plastic syringes[9] with flat, rubber-tipped plungers. These volumes were more reproducible than those measured by pipettes (in which the meniscus of packed blood cells cannot be seen) or by glass syringes (in which cells in the bevelled rim of the plunger obscure the exact volume of cells contained in the syringe).

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[8] Tris, tris(hydroxymethyl)aminomethane.

[9] 'Tomac' disposable syringes were purchased from American Hospital Supply.

(ii) Pretreatment of Cells

To prepare erythrocytes for efflux studies, washed cells were mixed with 1 volume of medium A containing radioactive nucleoside, and were allowed to equilibrate with the substrate during incubation in a shaking water bath for 30 minutes at 37°. The cells were collected by centrifugation for 15 minutes in a bench centrifuge at room temperature.

Cells used in uptake assays were preincubated in 2 volumes of medium A for 30 minutes at 37° and were collected by centrifugation. Except where noted, inhibitor-treated cells were prepared by incubating these erythrocytes in 2 volumes of medium A containing the inhibitor for a further 5 minutes at 37°, and centrifuging the cells through 5 additional volumes of medium; the large volume was used to dilute the inhibitor and minimise further reaction with the cells. Both control and inhibitor-treated cells were finally washed 3 times in 3 volumes of medium A and suspended to a concentration of 40% by volume.

(iii) Measurement of Rates of Transport

Radioactive substrates were used in all measurements of transport rates, and the velocity of nucleoside movement was calculated from the radioactivity which appeared in, or disappeared from, the incubation medium. In these studies, rapid separation of cells from medium was achieved by layering portions of assay mixtures onto dibutyl phthalate (DBP) and applying a centrifugal force. DBP is immiscible with water and has a density intermediate between that of erythrocytes and the incubation medium (57); upon centrifugation

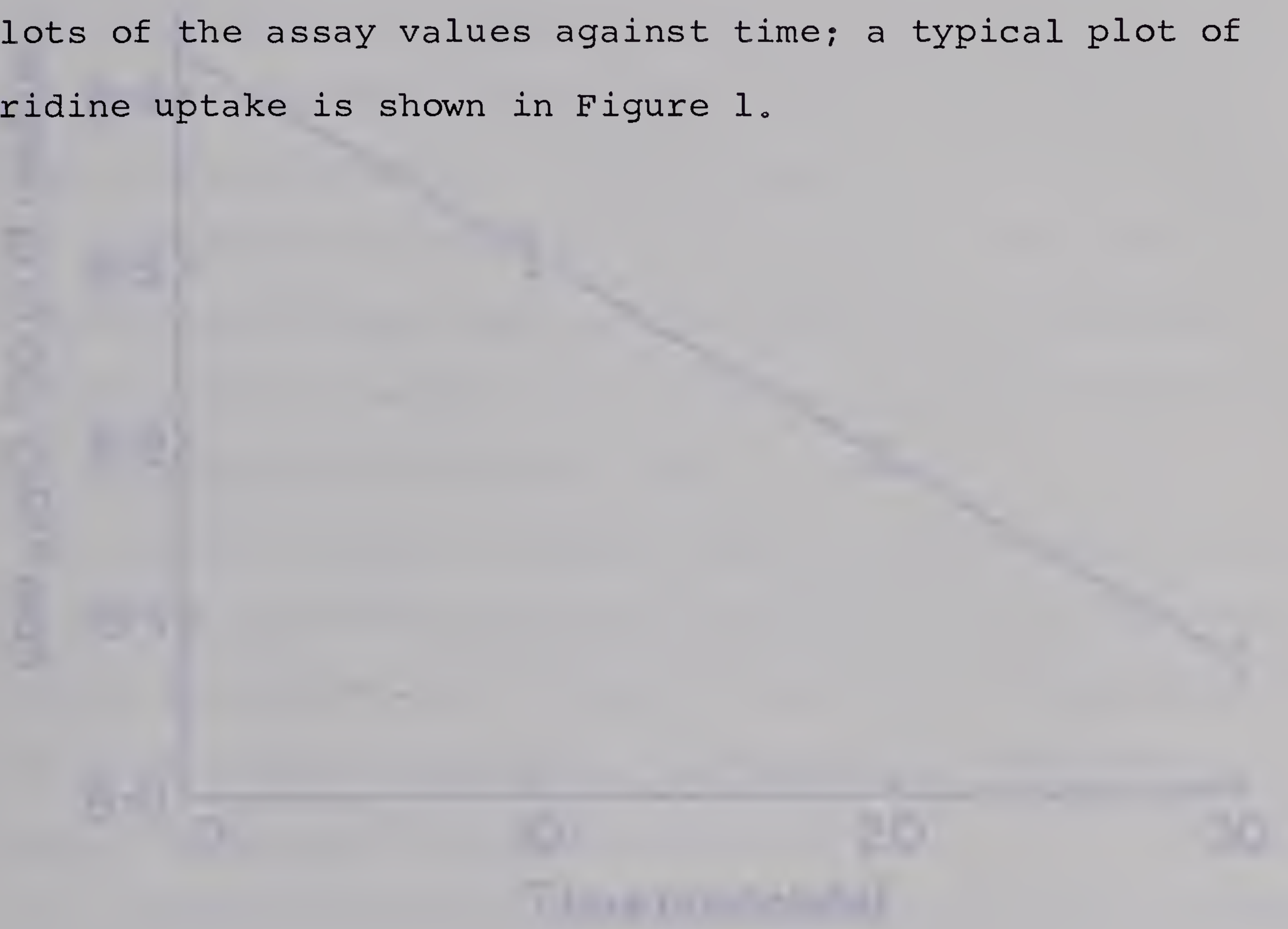
the cells pass rapidly through this liquid, leaving the medium above the DBP.

The incubation mixtures used in studies of nucleoside efflux contained 1.0 ml of cells which had been 'loaded' with radioactive substrate and 4.0 ml of medium A (with or without added inhibitor). Incubation mixtures consisting of 4.5 ml of a 40% cell suspension and 0.5 ml of radioactive substrate were employed in uptake assays. The incubations were carried out at specified temperatures in 25 ml flasks, with continuous magnetic stirring of the cell suspensions.

In both influx and efflux assays, samples (approximately 1 ml in volume) of the incubation mixtures were removed at particular time intervals, placed onto 5 ml portions of DBP contained in 30 x 100 mm tubes, and were spun at top speed for 1.5 minutes in bench centrifuges. Each centrifuge contained a single sample, and the centrifuges were switched on at exactly 10, 20 and 30 seconds after starting the reaction by addition of cells in exit assays, or by the addition of substrates in uptake assays. Radioactivities in 50 μl portions of each supernatant were determined by liquid scintillation counting using Bray's counting fluid (58). Haematocrits were determined for each incubation by the capillary method.

This assay was repeated at least 9 times for each estimation of the rate of transport of a nucleoside, and the counts per minute (cpm) in 50 μl of medium were averaged for each of the 3 time points. The deviations of the individual values from the mean were calculated; those which differed

from the mean by more than 2 standard deviation units were discarded and the results recalculated. The experiment was repeated if more than 2 values from 9 were rejected. The initial velocity of efflux or influx was determined from plots of the assay values against time; a typical plot of uridine uptake is shown in Figure 1.



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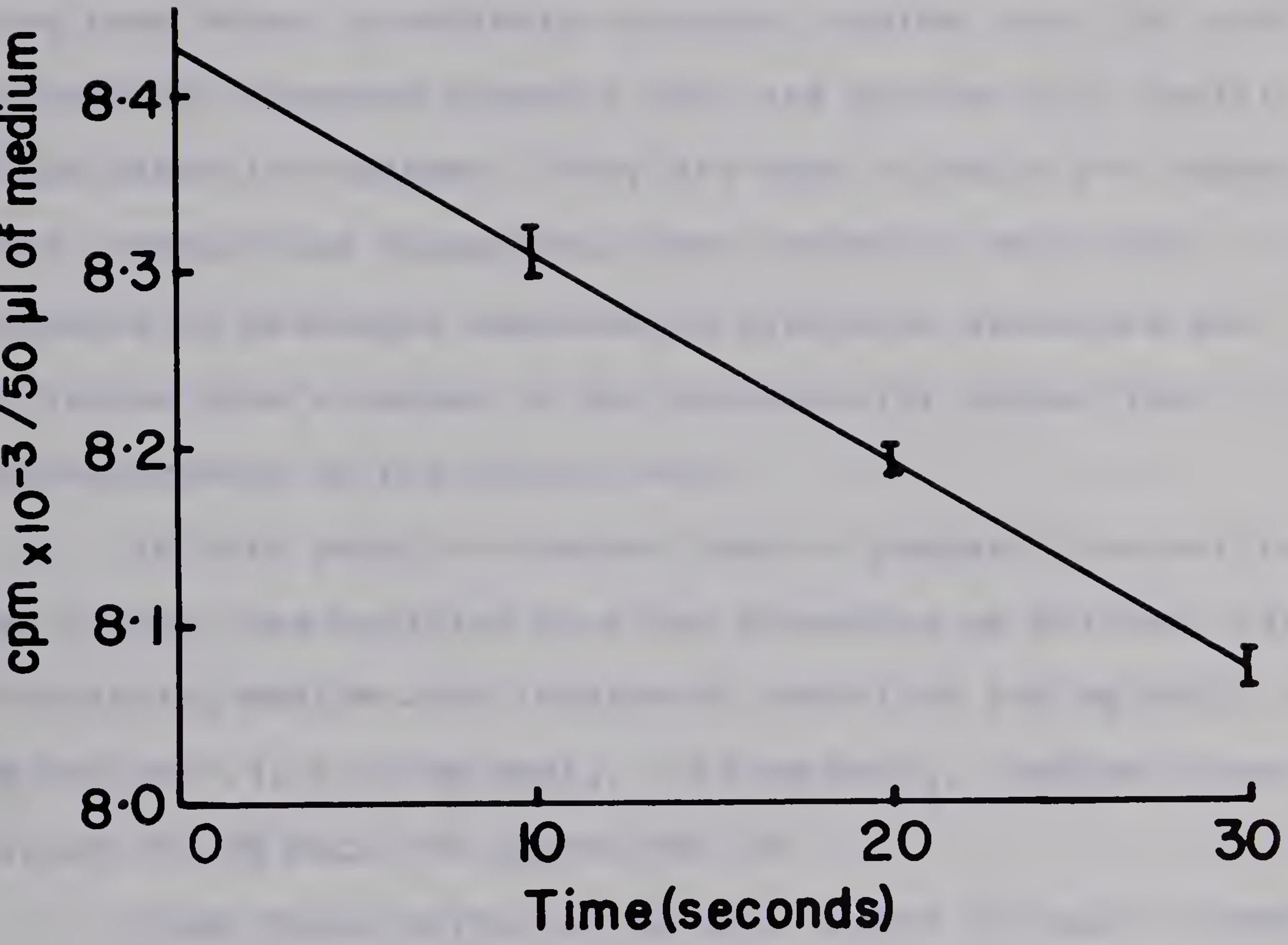

Figure 1: A typical plot of uridine uptake by erythrocytes. Uridine-2-^{14}C (specific activity, 5.71 x 10^4 cpm/μmole) was initially 2.6 m<u>M</u> in the media of replicate 5 ml cell suspensions (28.3%, by volume) at 25°. The standard deviation for the average of 9 individual determinations of each assay value is shown. From the rate of disappearance of radioactivity from the incubation media, the calculated velocity of uridine uptake was 0.63 μmoles/minute/ml of packed cells.

IV. The Preparation of 'Ghosts'

'Reconstituted ghosts', which are prepared by brief haemolysis of erythrocytes, have some permeability properties similar to those of intact cells. For instance, they have been shown to actively transport sodium ions (59) and to possess unchanged glycerol (60) and glucose (61) facilitated diffusion systems. They are more suitable for transport studies than haemoglobin-free 'ghosts', which are prepared by prolonged exposure to hypotonic solutions and no longer show a number of the permeability properties characteristic of the intact cell.

In this study the method used to prepare 'reconstituted ghosts' was modified from the procedure of Whittam (62). The washing medium used (medium B) contained 140 m$\underline{M}$ NaCl, 18 m$\underline{M}$ TES pH 7.4, 2.25 m$\underline{M}$ $MgCl_2$, 2.25 m$\underline{M}$ $CaCl_2$. Medium C contained 700 m$\underline{M}$ NaCl, 90 m$\underline{M}$ TES pH 7.4.

Fresh human erythrocytes were washed 3 times in medium B and the cells were then suspended in 5 volumes of 4 m$\underline{M}$ $MgCl_2$, 4 m$\underline{M}$ $CaCl_2$. After 3 minutes of haemolysis, sufficient medium C was added to restore the suspension to the same tonicity as medium B, and the suspension was shaken gently for 30 minutes at 37°. The cells were finally washed 3 times in medium B; this procedure yielded a high recovery of reddish 'ghosts'. Mg^{2+} and Ca^{2+} were essential to prevent secondary haemolysis during the final washing of the 'ghosts'.

RESULTS

I. Uridine Metabolism

Previous investigations of nucleoside transport have been difficult to interpret because of the intracellular metabolism of the permeants (Introduction, III (ii)). Evidence obtained in this laboratory has indicated that uridine is not metabolised by erythrocytes of various animals, including man (39); this conclusion was further investigated, since studies of nucleoside transport would be greatly facilitated by use of a non-metabolised permeant.

To determine whether human erythrocytes are able to support ribosyl transfer reactions involving uridine, the ability of cell suspensions to catalyze the phosphorolysis of uridine, the inosine-dependent synthesis of uridine from uracil, and uridine-uracil exchange was tested. The results, summarized in Table I, indicate that the cells did not catalyse cleavage, synthetic or exchange reactions involving uridine.

In a separate experiment, a suspension of washed erythrocytes (40% by volume) was incubated with 4 m<u>M</u> uridine-2-^{14}C at 37^{o} for 30 minutes, and a neutralised perchloric acid extract of the cells was analyzed by paper chromatography. No radioactivity was recovered as uracil, confirming the observation that human erythrocytes lack uridine phosphorylase. The absence of radioactivity accompanying the uridine

phosphate 'carriers' on the chromatograms indicated that these cells also lack uridine kinase. These data strongly suggest that human erythrocytes are unable to metabolise uridine.

TABLE I

Ability of human erythrocytes to conduct ribosyl transfer reactions involving uridine[a]

Substrates (1.0 mM)	% Distribution of recovered radioactivity	
	Uracil	Uridine
Uridine-2-^{14}C	0.2	99.8
Uracil-2-^{14}C + inosine	99.6	0.4
Uracil-2-^{14}C + uridine	99.7	0.3

[a] See Materials and Methods, II for the assay procedure.

II. Nucleoside Transport

Criteria which may be used to identify a particular transport mechanism have been discussed (Introduction, II). In the following studies, uridine has been used to investigate the mechanism of nucleoside transport in human erythrocytes.

(i) Evidence against Active Transport

Active transport systems may be identified experimentally by their ability to accumulate substrates against a concentration gradient and by their dependence upon energy metabolism.

In order to determine the equilibrium distribution of uridine across the erythrocyte membrane, washed cells were suspended to 42%, by volume, in medium A containing (a) no addition, (b) 4.5 μc of inulin-carboxyl-^{14}C (a polysaccharide which is excluded from erythrocytes) and (c) 1.6 m$\underline{M}$ uridine-2^{14}C (specific activity 2.08 x 10^5 cpm/μmole). These 20 ml suspensions were incubated in a shaking water bath for 30 minutes at 37° before separation of the cells from the media by centrifugation for 15 minutes at approximately 3500 rpm in a bench centrifuge. The concentrations of extracellular inulin and uridine in (b) and (c), respectively, were calculated from radioactivity in portions of the supernatants of these suspensions. Extracellular water in the cell pellets was estimated from the radioactivity due to inulin-carboxyl-^{14}C in acid extracts of the cells of suspension (b); intracellular water was determined by drying measured portions of packed cells from suspension (a) to constant weight at 110°,

calculating the total water content in these pellets and subtracting the value found for extracellular water. The concentration of uridine in the intracellular water was calculated from the amount of uridine in acid extracts of portions of packed cells from suspension (c) and from the determined values of 68% for intracellular water and 8% for extracellular water in the cell pellets. The data obtained in this experiment, and the calculation of the distribution of uridine across the erythrocyte membrane, are summarised in Appendix I; they show that the concentration of uridine was 1.06 mM in the intracellular water and 0.96 mM in the incubation medium. This apparent lack of accumulation within the cells indicates that uridine uptake is not mediated by an active transport system.

To test this conclusion, 'reconstituted ghosts' were prepared in the absence of glucose, and several properties of these 'depleted' cells and the intact cells from which they were derived were compared. The results, listed in Table II, show that the initial velocity of uridine uptake was unaffected by the total removal of ATP, which occurred during the preparation of 'ghosts'; this is further evidence that uridine transport is independent of metabolic energy. These data also suggest that uridine transport is accomplished by a component of the erythrocyte membrane, since a severe depletion of the intracellular contents (indicated by the removal of a large part of the haemoglobin and of several enzyme activities) had no effect on the rate of uridine uptake.

TABLE II

Comparison of properties of intact erythrocytes and of erythrocyte 'ghosts'

Property	Intact erythrocytes	Erythrocyte 'ghosts'
Uridine uptake (μmoles/minute/ml of intact cells or the equivalent number of 'ghosts')[a]	0.73	0.73
ATP concentration (μmoles/ml of intact cells or of 'ghosts')[b]	0.40	0
Haemoglobin level (% of intact cells)[c]	100	29
Rate of ribosyl transfer reactions (% of intact cells)[d]		
(i) Phosphorolysis of inosine	100	50
(ii) Guanosine-supported synthesis of inosine	100	37

NOTE: Blood was used 16 hours after collection; the various properties were compared with intact erythrocytes and with 'ghosts' prepared from the same batch of cells.

In measurement of uridine uptake, 'ghosts' were separated from medium by centrifugation of the cells through dibutyl phthalate containing 20% of di(2-ethylhexyl) sebacate[10].

[a] Uptake of 2.5 m$\underline{M}$ uridine-2-^{14}C was measured at 30°.

[b] ATP was assayed by the phosphoglycerate kinase method (63).

[c] Haemoglobin levels were determined by the cyanomethaemoglobin method (64).

[d] The substrates employed were (i), 1.0 m$\underline{M}$ inosine-8-^{14}C and (ii) 1.0 m$\underline{M}$ hypoxanthine-8-^{14}C plus 1.0 m$\underline{M}$ guanosine.

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[10] di(2-ethylhexyl) sebacate, ('Octoil-S'), was purchased from Consolidated Vacuum Company.

(ii) Evidence against Simple Diffusion

It has been found experimentally that the entry of substances into cells by simple diffusion is characterised by temperature-dependence with a Q_{10} value of less than 1.5, by non-saturating influx kinetics and by an absence of competition between related permeants for transport.

The temperature-dependence of uridine uptake was examined by measuring the rate of influx at 4 different temperatures. The results, listed in Table III, show that the Q_{10} value for the influx of uridine is approximately 2. This is in the range which has been observed for mediated transport (13), and suggests that a process other than simple diffusion may be involved in the movement of uridine across the erythrocyte membrane.

To determine whether the transport process is saturable, the rate of uridine uptake was measured over a range of initial concentrations from 0.2 m$\underline{M}$ to 2.5 m$\underline{M}$. The assays were carried out at 15°, since at higher temperatures low (0.1 - 0.5 m$\underline{M}$) concentrations of uridine approached an equilibrium distribution rapidly and plots of influx against time of incubation were not linear at 30 seconds. Figure 2 shows that the transport system was saturated at high levels of uridine; this effect is not consistent with the influx kinetics of simple diffusion, but it is predicted for permeation by facilitated diffusion.

Formycin B, an analogue of inosine which is not phosphorolysed by human erythrocytes (65), was used to determine the effect of a second nucleoside on the rate of uridine influx. Figure 3 is a Lineweaver-Burk plot of uridine uptake

TABLE III

Effect of temperature on uridine uptake

Temperature (°C)		Uridine uptake[a] (µmoles/minute/ml of cells)	Q_{10}[b]
(i)	15	0.36	1.83
	25	0.66	
(ii)	27	0.73	1.93
	37	1.41	

[a] Uridine-2-^{14}C was initially 2.6 m<u>M</u> in the incubation media.

[b] $$Q_{10} = \frac{\text{uridine uptake at } (T + 10)^{\circ}}{\text{uridine uptake at } T^{\circ}}$$

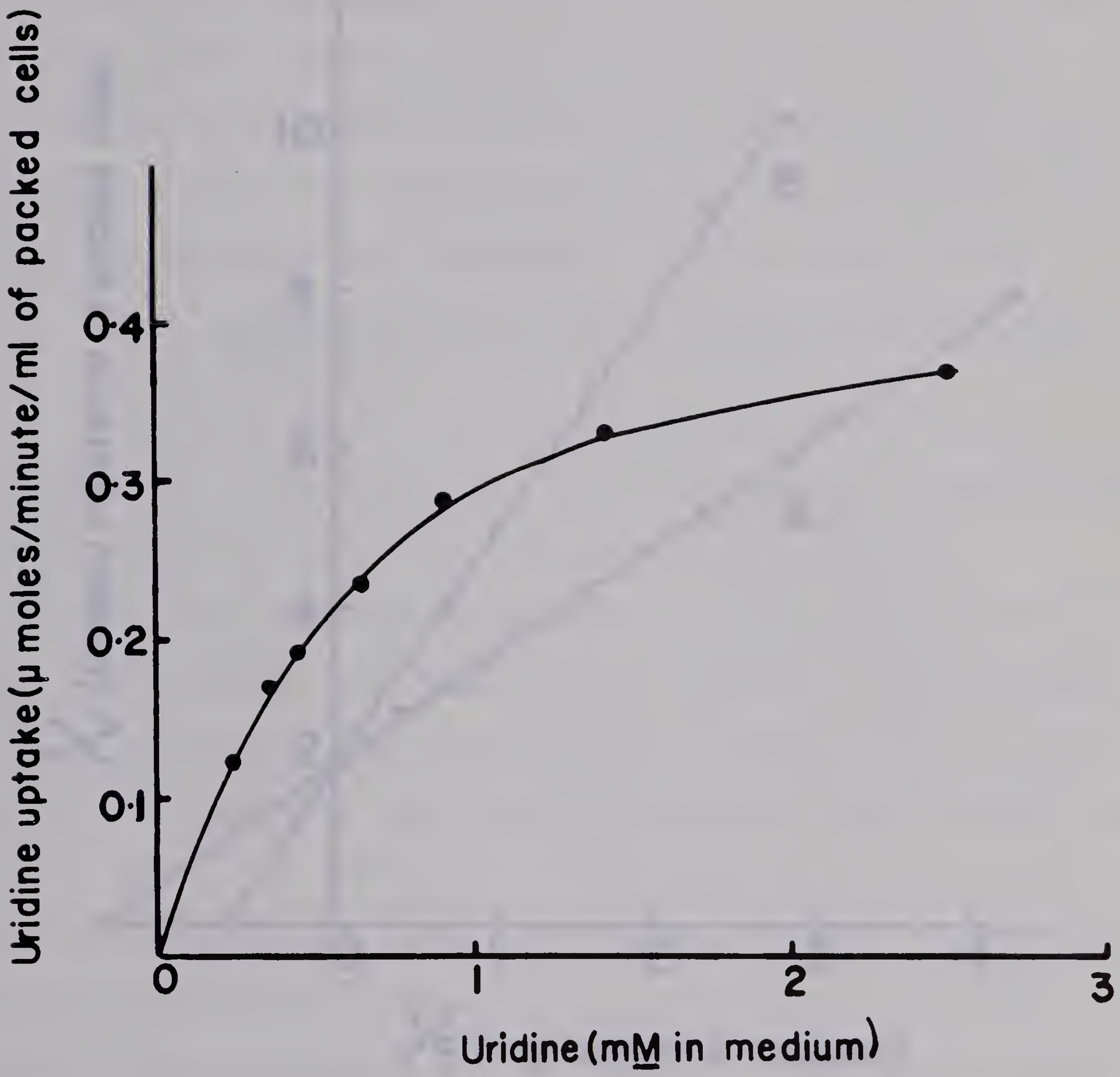

Figure 2: Effect of concentration on uridine uptake at 15°.

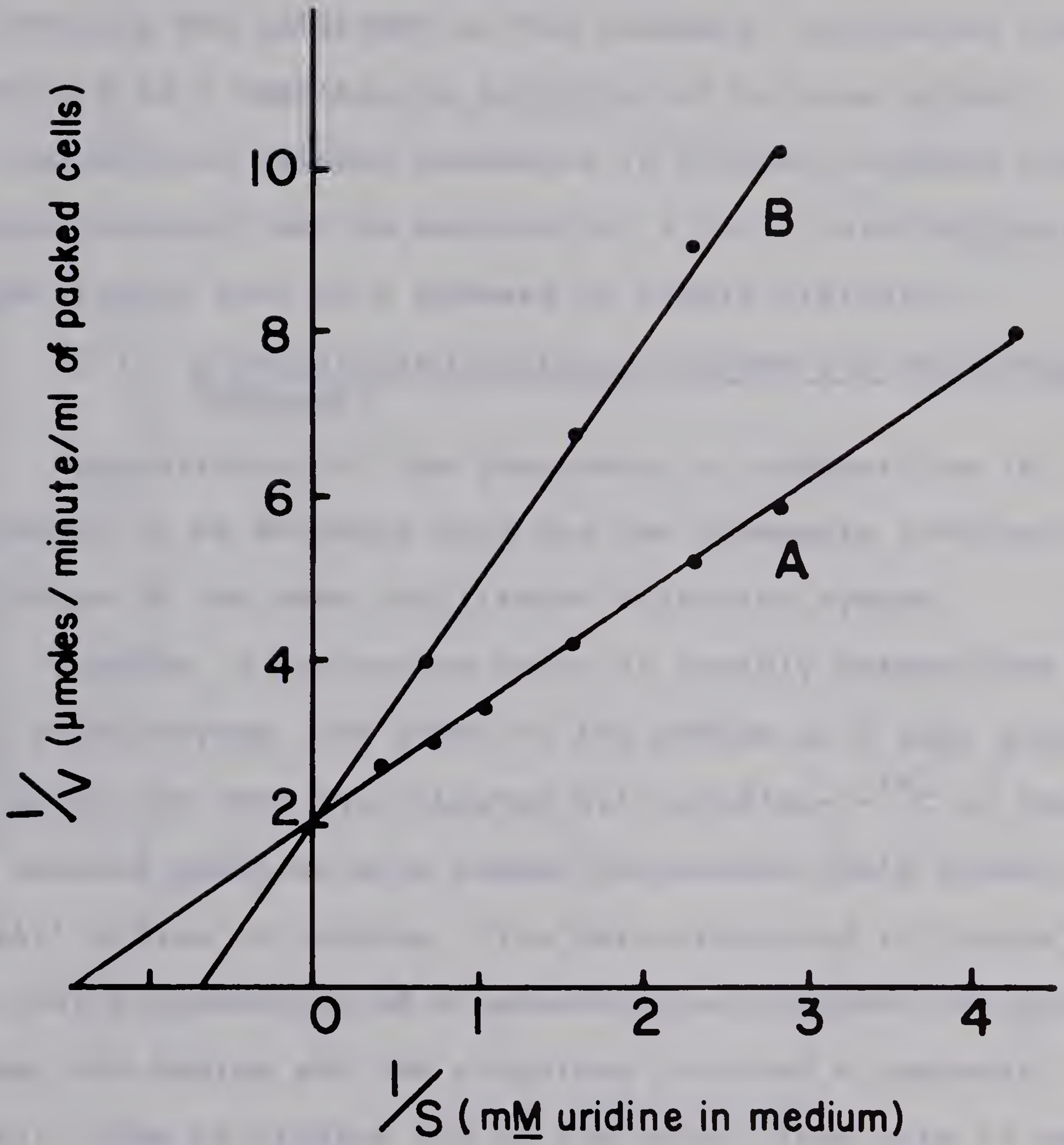

Figure 3: Competitive inhibition of uridine uptake by formycin B. Uridine uptake was measured at 15° from medium containing uridine-2-^{14}C (A) and from medium containing uridine-2-^{14}C plus 0.35 m*M* formycin B (B). Values for the slopes and intercepts, calculated by the method of least squares, were respectively 1.38 and 2.02 in (A) and 2.96 and 2.04 in (B).

in the presence and absence of 0.35 mM formycin B; the added nucleoside increased the slope of the reciprocal plot without changing the intercept on the ordinate, indicating that formycin B is a competitive inhibitor of uridine uptake. This competition between permeants is further evidence that uridine transport may be mediated by a facilitated diffusion system, rather than by a process of simple diffusion.

(iii) A Facilitated Diffusion System for Nucleoside Transport

Demonstration of the phenomenon of counterflow is considered to be evidence that the two permeants involved are substrates of the same facilitated diffusion system.

Inosine, a nucleoside which is rapidly metabolised by human erythrocytes, was added to the medium of a cell suspension which had been equilibrated with uridine-2-^{14}C to determine whether addition of a second nucleoside could promote an 'uphill' efflux of uridine. The data presented in Figure 4 show that introduction of a concentration gradient of inosine between the medium and the cytoplasm produced a temporary 'uphill' flow of uridine out of the cells; from this it can be concluded that these permeants share a common transport 'carrier'. The flow of uridine back into the cells after approximately 4 minutes is probably due to disappearance of the inosine gradient.

The specificity of the transport system was investigated by measuring the effect of various extracellular compounds on the rate of uridine efflux from cells containing 10 mM uridine-2-^{14}C. The results are plotted in Figures 5A, 5B and 5C.

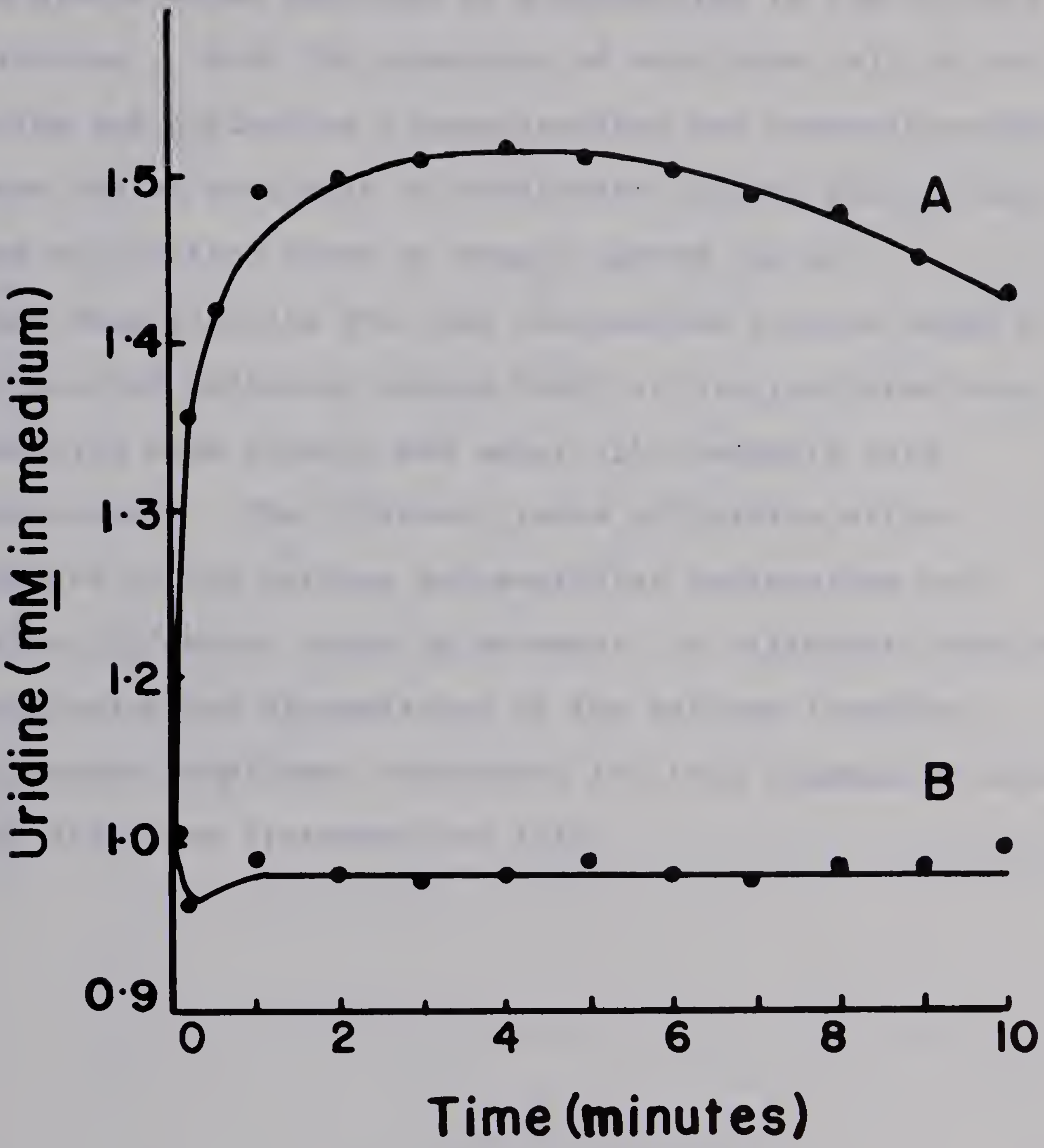

Figure 4: 'Uphill' transport of uridine. Replicate 16 ml cell suspensions (45% by volume) were incubated at 37° for 30 minutes to an equilibrium concentration of 1.01 mM uridine-2-^{14}C in the medium and in the intracellular water. Uridine efflux was measured in duplicate at 20° after addition of inosine to an initial concentration of 4.0 mM in the medium of suspension (A) and after addition of an equal volume (0.4 ml) of medium to suspension (B).

Table IV lists the concentrations of uridine in the media one minute after addition of erythrocytes to the various solutions. With the exception of orotidine, all of the purine and pyrimidine ribonucleosides and deoxyribonucleosides tested were able to accelerate uridine efflux, but none of the free bases or sugars tested did so. These data indicate that the nucleosides studied share a facilitated diffusion system which is distinct from that mediating base (20-23) and sugar (1) transport into erythrocytes. The different rates of uridine efflux produced by the various extracellular nucleosides may reflect different rates of movement, or different rates of association and dissociation of the various 'carrier'-nucleoside complexes; precedents for this suggestion have been discussed (Introduction III).

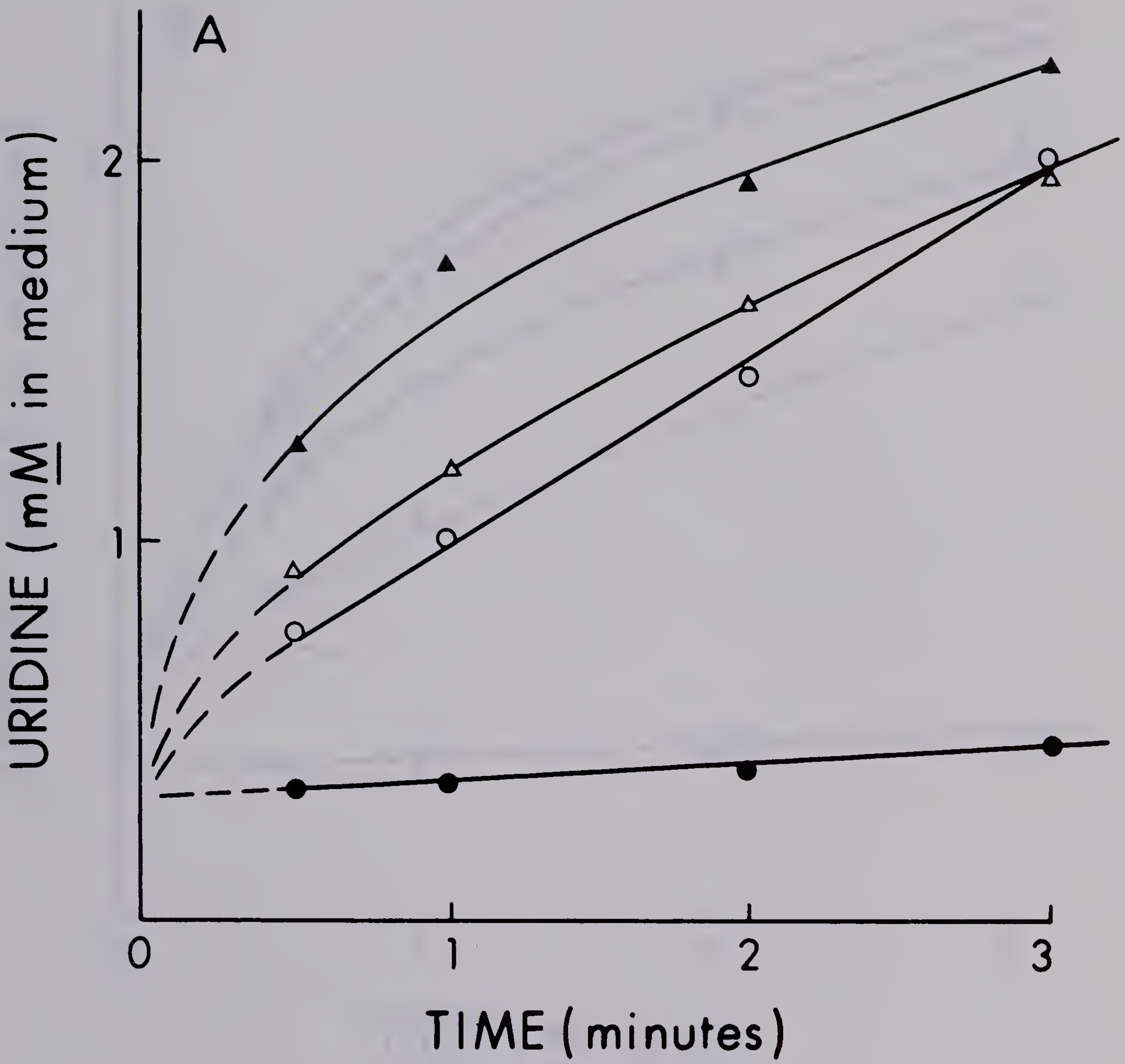

Figure 5A: Effect of extracellular permeants on uridine efflux. Cell suspensions consisted of 3.0 ml of erythrocytes, which had been preincubated to a concentration of 10.5 m<u>M</u> uridine-2-^{14}C in the intracellular water, and 6.0 ml of medium containing 10 m<u>M</u> permeants. Uridine in the medium was measured after the indicated times of incubation at 20°. No significant difference was observed between efflux into medium alone and into medium containing hypoxanthine or uracil (●). Efflux was accelerated when the medium contained formycin B (○), uridine (▲) and inosine (△).

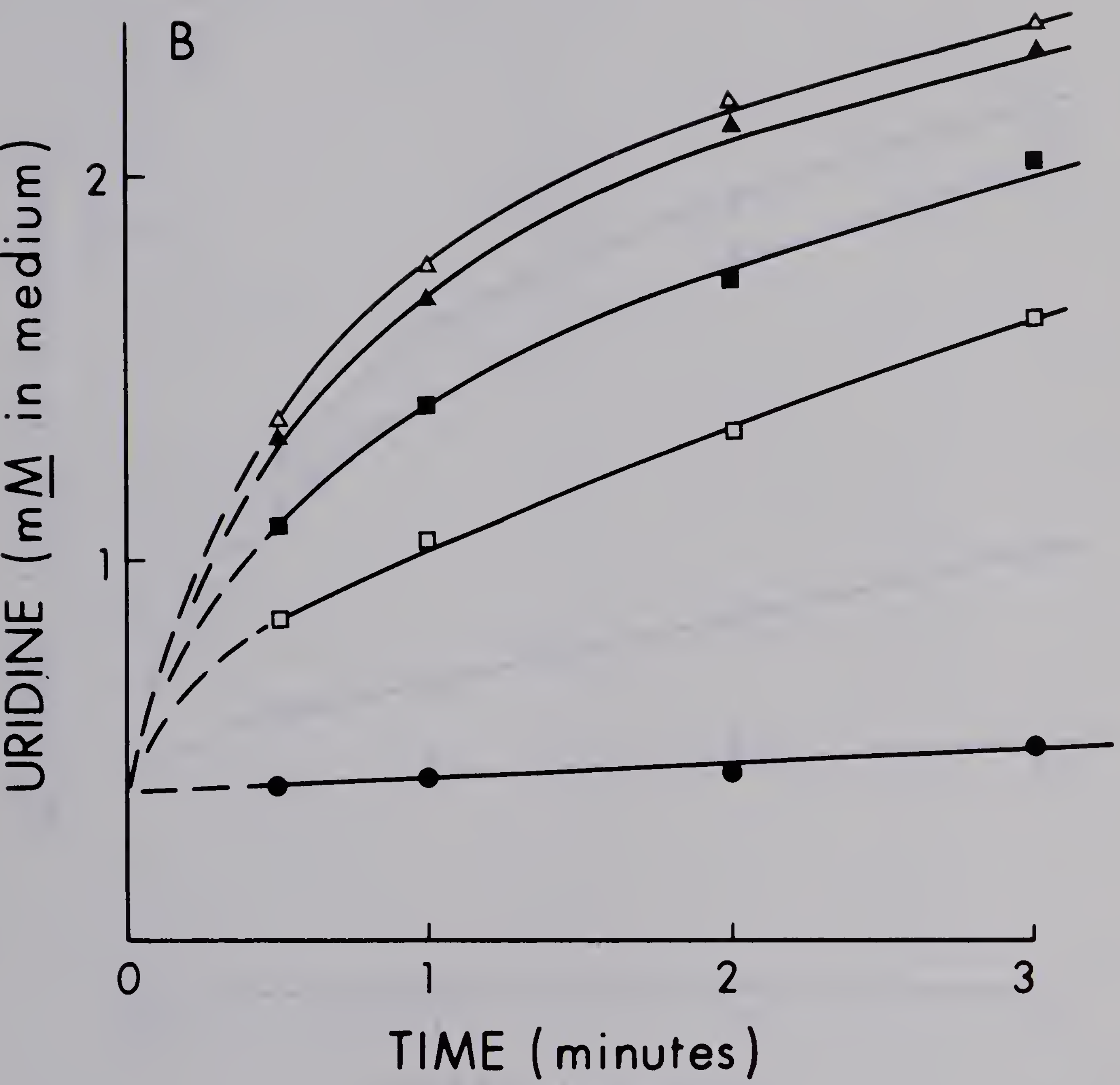

Figure 5B: Effect of extracellular permeants on uridine efflux. The assay conditions have been described in Figure 5A. No significant difference was observed between efflux into medium alone and into medium containing cytosine or adenine (●). Efflux was accelerated when the medium contained adenosine (□), deoxyuridine (■), cytidine (▲), and guanosine (△).

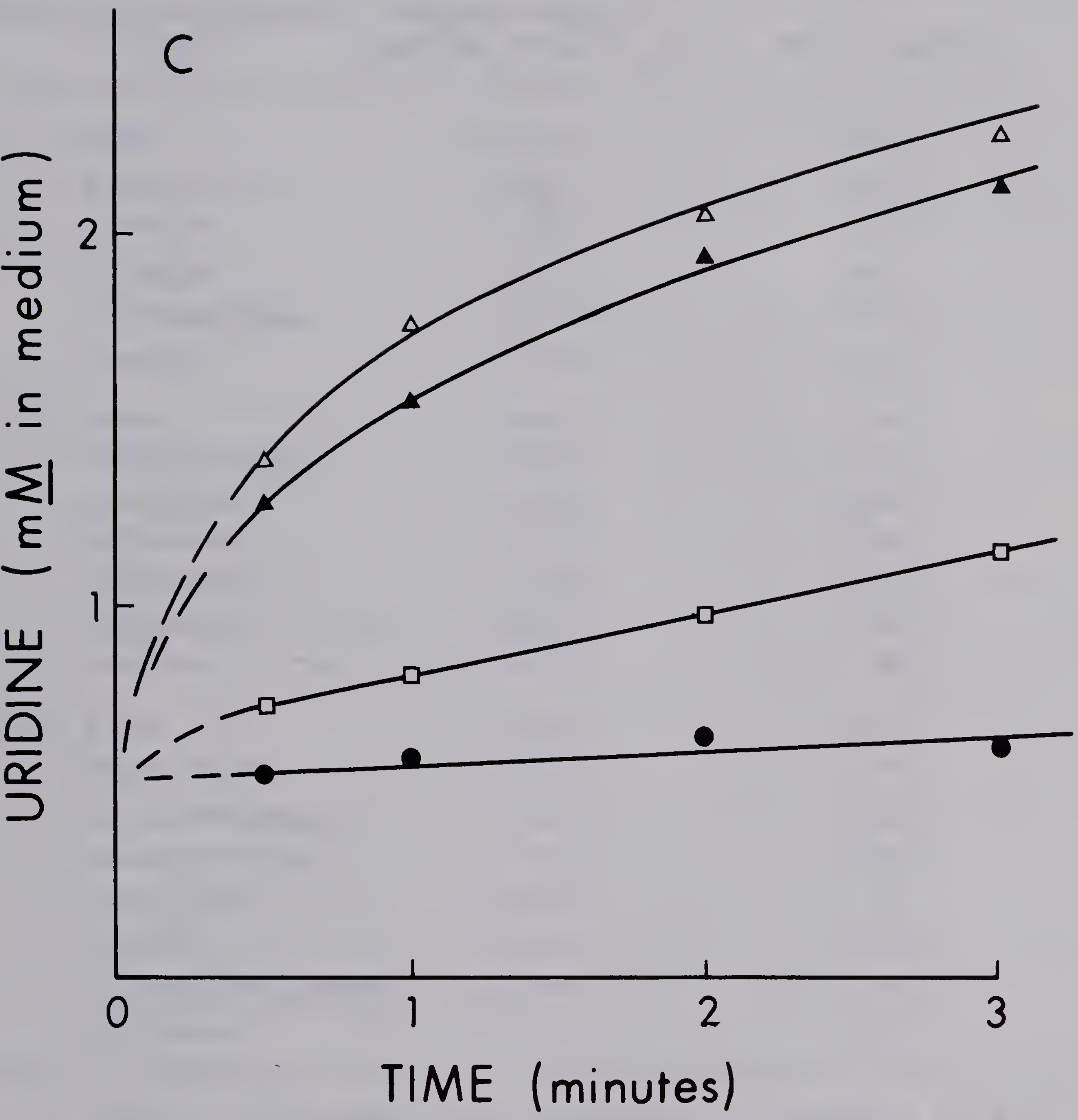

Figure 5C: Effect of extracellular permeants on uridine efflux. The assay conditions have been described in Figure 5A. No significant difference was observed between efflux into medium alone and into medium containing 2-deoxy-D-glucose, D-ribose or orotidine (●). Efflux was accelerated into deoxyadenosine (□), thymidine (▲) and deoxycytidine (△).

TABLE IV

Effect of extracellular permeants on uridine efflux

Additions to medium (10 m*M*)		Uridine (m*M* in medium after 1 min)	% of control
(i)	None	0.367	100
	Formycin B	1.007	274
	Inosine	1.185	323
	Uridine	1.527	416
	Hypoxanthine	0.373	102
	Uracil	0.370	101
(ii)	None	0.419	100
	Deoxyuridine	1.404	335
	Cytidine	1.682	401
	Adenosine	1.038	248
	Guanosine	1.760	420
	Cytosine	0.429	102
	Adenine	0.413	99
(iii)	None	0.576	100
	Thymidine	1.558	270
	Deoxyadenosine	0.801	139
	Deoxycytidine	1.751	297
	Orotidine	0.556	97
	AICAR[11]	0.891	154
	2-Deoxy-D-glucose	0.537	93
	D-ribose	0.536	93

NOTE: Duplicate cell suspensions consisted of 3.0 ml of erythrocytes (preincubated to a concentration of 10.5 m*M* uridine-2-^{14}C in the intracellular water) and 6.0 ml of medium A, containing one of the permeants listed. Uridine-2-^{14}C was measured in the media after various times of incubation at 20° as described in Materials and Methods, III (iii); values after 1 minute of incubation are shown above.

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[11] AICAR, 5-amino-4-imidazolecarboxamide ribonucleoside.

III. Inhibition of Nucleoside Transport by a 6-(Alkylmercapto)-purine Ribonucleoside

It has been proposed that a number of 6-alkylmercapto derivatives of purine ribonucleoside and 2-aminopurine ribonucleoside may be competitive, irreversible inhibitors of nucleoside transport in human erythrocytes (40-42). This possibility has been examined using NBzTGR, the most potent inhibitor of the series.

To determine whether the effect of NBzTGR on the transport system is reversible, the ability of inhibitor-treated cells to transport uridine was examined after more or less extensive washing of these cells in medium which lacked added inhibitor. Cells were incubated for 5 minutes at 37° with 10^{-6} $\underline{M}$ NBzTGR and were collected by centrifugation through 5 volumes of medium A. Portions of the cells were washed once, twice or three times by suspension in 3 volumes of medium A, incubation for 5 minutes at 37°, and recentrifugation. In subsequent assays,[12] no uridine uptake by the unwashed or washed cells could be detected, indicating that the inhibition of nucleoside transport by NBzTGR is not readily reversed.

It has been observed that specific irreversible inhibitors of enzymes usually react with their 'target' proteins in a time- and temperature-dependent fashion (66). The possibility that NBzTGR inactivates the nucleoside transport system of human erythrocytes in an analogous manner has been examined. The rate of uridine transport into erythrocytes was measured after incubation of the cells with 3×10^{-7} $\underline{M}$

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[12] Uridine-2-^{14}C was initially 2.5 m$\underline{M}$ in the incubation media and influx was followed at 30°C.

NBzTGR for 5 minutes at various temperatures. From Figure 6A, it can be seen that the rate of uridine uptake into cells preincubated with inhibitor at 2° was considerably higher than that into cells exposed to inhibitor at 15° or 30°. The data in Figure 6B show that there was a linear and reciprocal relationship between the rate of uridine influx and the length of preincubation of the cells at 2° with $3 \times 10^{-7}\underline{M}$ NBzTGR. From these observations it is concluded that the inactivation of the nucleoside transport system by NBzTGR is a time- and temperature-dependent process.

The possibility that NBzTGR may be a competitive inhibitor of the nucleoside transport system of human erythrocytes could not be examined directly, since the usual tests for competition, such as the construction of Lineweaver-Burk plots, are difficult to apply with an apparently irreversible inhibitor (66). An alternative procedure was therefore employed which determined whether the addition of a nucleoside to the medium could protect the transport system against inactivation by NBzTGR. Washed erythrocytes were incubated for 5 minutes at 37° with various combinations of uridine and NBzTGR, and their ability to transport uridine was subsequently measured. The results, which are presented in Figure 7, indicate that high concentrations of uridine were able to decrease the effect of NBzTGR on the transport system. These data suggest that the inhibitor may compete with uridine for a common binding site on the nucleoside transport system.

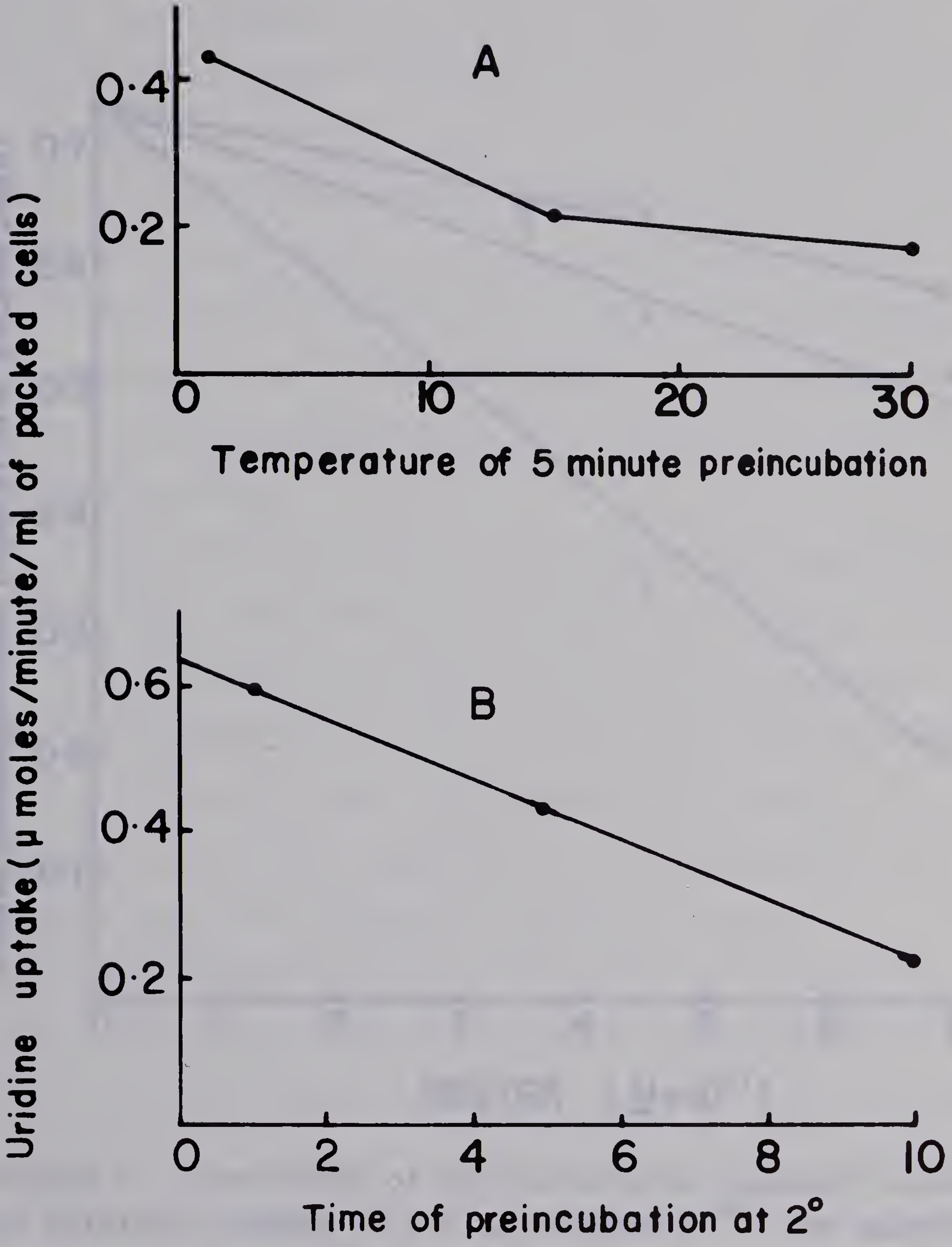

Figure 6: Time- and temperature-dependence of transport inhibition by 2-amino-6-(p-nitrobenzylmercapto) purine ribonucleoside. Erythrocytes were preincubated with 3×10^{-7} $\underline{M}$ NBzTGR under the specified conditions of time and temperature. Uptake of 2.5 m$\underline{M}$ uridine into these cells was measured at 30°.

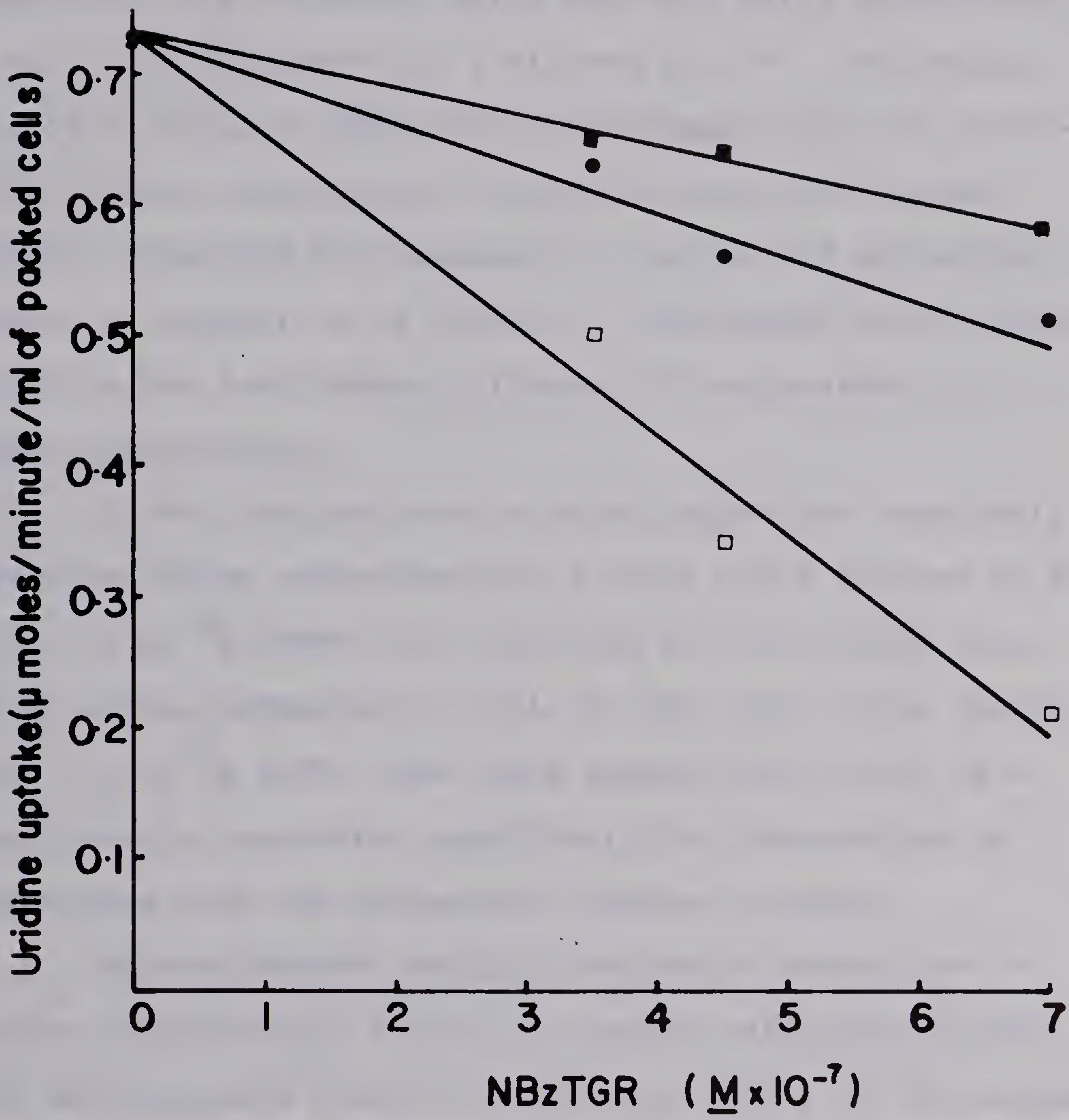

Figure 7: Protection of the nucleoside transport system with uridine. Uptake of 2.5 m<u>M</u> uridine-2-^{14}C was measured at 30° after preincubation of erythrocytes with 2-amino-6-(<u>p</u>nitrobenzylmercapto) purine ribonucleoside (□), with NBzTGR plus 16 m<u>M</u> uridine (●), and with NBzTGR plus 62 m<u>M</u> uridine (■).

The specificity of the inhibition by NBzTGR was investigated by comparing the rates of uptake of various compounds into untreated cells and into cells preincubated with 6 x 10^{-6}M NBzTGR for 5 minutes at 37°. The results, listed in Table V, show that pretreatment with the nucleoside analogue specifically inhibited nucleoside uptake without affecting the transport of purine and pyrimidine bases, of sugars, or of leucine. Thus, NBzTGR specifically inhibits the facilitated diffusion of nucleosides in human erythrocytes.

It was observed that uridine uptake was completely inhibited after preincubation of cells for 5 minutes at 37° with 5 x 10^{-5}M BzTGR, but continued at the control rate (0.73 μmoles/minute/ml of cells at 30°) into cells treated with 5 x 10^{-5}M BzTG. These data suggest that there is a considerable structural specificity for interaction of inhibitors with the nucleoside 'carrier' system.

Because NBzTGR inhibits nucleoside metabolism in intact erythrocytes, but not in broken cell preparations, it has been proposed that the inhibitory effect of the nucleoside analogue is a result of interaction with the cell membrane (40). This possibility was supported in this study by the demonstration (a) that uridine uptake into 'reconstituted ghosts' occurred at the same rate as that into intact erythrocytes (Table II), and (b) that preincubation of the 'ghosts' with 9 x 10^{-6}M NBzTGR for 5 minutes at 37° completely inhibited uridine influx.

Inhibitors of facilitated diffusion systems characteristically block both the influx and efflux of permeants (1).

TABLE V

Specificity of transport inhibition by 2-amino-6-(p-nitrobenzylmercapto)purine ribonucleoside

Permeant	Influx of permeants (μmoles/minute/ml of cells)[a] (i) Untreated erythrocytes	Influx of permeants (μmoles/minute/ml of cells)[a] (ii) Inhibitor-treated erythrocytes
Uridine -2-^{14}C	0.7	0
Inosine -8-^{14}C	0.7	0
Cytidine -2-^{14}C	0.6	0
Uracil-2-^{14}C	1.3	1.3
Hypoxanthine -8-^{14}C	1.1	1.1
Cytosine -2-^{14}C	0.8	0.8
D-glucose -U-^{14}C	1.7	1.6
L-leucine -U-^{14}C	0.3	0.3

NOTE: The same blood (stored for 25 days in ACD medium) was used in all assays. Washed cells were preincubated either with medium A (i), or with medium A containing 9 x 10^{-6}M 2-amino-6-(p-nitrobenzylmercapto) purine ribonucleoside (ii).

[a] Permeants were initially 2.5 mM in the incubation media and uptake was measured at 30°, except for D-glucose (6.0 mM, 5°) and L-leucine (0.7 mM, 30°).

When erythrocytes containing 10 m$\underline{M}$ uridine-2-^{14}C were collected and suspended in medium A, the initial rate of efflux of uridine was rapid (0.86 μmoles/minute/ml of cells at 30°), whereas no efflux was observed if the cells were suspended in medium A containing 9 x 10$^{-6}\underline{M}$ NBzTGR. The observation that NBzTGR inhibits nucleoside efflux was confirmed in an experiment in which cells containing 10 m$\underline{M}$ uridine-2-^{14}C were resuspended in medium A in the presence or absence of 9 x 10$^{-6}\underline{M}$ NBzTGR and incubated for 10 minutes at 30°. Analysis of the distribution of radioactivity between cells and medium, which is presented in Table VI, showed that less than 6% of the radioactivity had left the inhibitor-treated erythrocytes, while in the control cells, uridine had reached an equilibrium distribution across the cell membrane. These data suggest that uridine has only a very slight ability to diffuse passively across the erythrocyte membrane when the nucleoside 'carrier' system is inactivated.

TABLE VI

Inhibition of uridine efflux with 2-amino-6-(p-nitrobenzylmercapto)purine ribonucleoside

Length in incubation (minutes)	Concentration of inhibitor (M)	Distribution of recovered radioactivity % in medium	% in cells
0	0	8	92
10	0	83	16
10	9×10^{-6}	14	86

NOTE: Duplicate incubation mixtures consisted of 1.0 ml of erythrocytes (preincubated to a concentration of 10 mM uridine-2-^{14}C in the intracellular water) and 4.0 ml of medium, with or without added 2-amino-6-(p-nitrobenzylmercapto)purine ribonucleoside. The suspensions were incubated in a shaking water bath at 30°, and cells and medium were separated by centrifugation for 15 minutes at approximately 3500 rpm in a bench centrifuge. Radioactivities in the media were measured by liquid scintillation counting of portions of the supernatants; portions of neutralised acid extracts of cells were counted to obtain radioactivity in the cell pellet.

DISCUSSION

Previous investigations of nucleoside transport have indicated that a mediated process may be involved in the movement of nucleosides across the outer membranes of a variety of cells (Introduction, III (ii)). However, most of these studies have been difficult to interpret in terms of a transport mechanism because the sampling techniques employed were not rapid enough to allow measurement of the initial rates of nucleoside transport, and because characteristic properties of the transport process may have been obscured by intracellular metabolism of the permeants.

In this study of nucleoside transport in human erythrocytes, a technique which permitted a rapid separation of cells from medium was used to measure the initial rates of influx and efflux of radioactive nucleosides. Samples of incubation mixtures were removed at 10 second intervals, layered onto DBP, and the cells were centrifuged through this liquid; the medium, which remained above the DBP, was subsequently analyzed for the appearance or disappearance of radioactivity.

The ability of human red blood cells to metabolise uridine was investigated during a search for a non-metabolisable permeant for transport studies. It was found that this nucleoside is not a substrate for phosphorolytic, synthetic or exchange reactions in human erythrocytes; these observations confirm the earlier report by Sandberg _et al_. (67) that haemolysates of human erythrocytes do not phosphorolyse

uridine or cytidine, and are consistent with the report by Paterson and Simpson (39) that, of red blood cells from a number of species, only rabbit erythrocytes metabolise uridine. Evidence indicating that human erythrocytes lack uridine kinase was also obtained, and this observation is supported by the finding of Bishop *et al*. (68), and of Bartlett (69) that the concentrations of uridine nucleotides in human erythrocytes are extremely low. This inability of human erythrocytes to metabolise uridine greatly simplified transport studies.

The characteristics of the various types of membrane transport processes which were discussed above (Introduction, II) suggested a number of experiments to identify the mechanism of nucleoside transport in human erythrocytes.

It was found that uridine had equilibrated between the medium and the intracellular water of cell suspensions incubated for 30 minutes with uridine-2-^{14}C; thus, no evidence for accumulative uptake of uridine in human erythrocytes was obtained. The influx of uridine was shown to occur at the same rate with intact erythrocytes and erythrocyte 'ghosts', indicating that uridine transport is independent of metabolic energy. From these facts, it is concluded that an active transport system is not involved in the movement of uridine into human erythrocytes.

Passive transport of nucleosides is also unlikely, since uridine uptake was shown to be a saturable process, and formycin B was found to be a competitive inhibitor of

uridine influx; these kinetic characteristics are consistent with the properties of facilitated diffusion systems but are not consistent with transport by simple diffusion. Uridine uptake was found to be temperature-dependent, with a Q_{10} of approximately 2, and this supports the evidence that nucleoside transport may be a mediated process.

Efflux of uridine against a concentration gradient was caused by addition of inosine to the medium of a cell suspension which had been equilibrated with uridine. This demonstration of counterflow indicated that the movement of inosine and uridine is mediated by the same facilitated diffusion system.

Further counterflow experiments showed that the nucleoside transport system has broad substrate specificity; with the exception of orotidine, all of the purine and pyrimidine ribonucleosides and deoxyribonucleosides tested were able to accelerate the efflux of uridine, indicating that a common facilitated diffusion system is utilized by this group. Different nucleosides stimulated uridine efflux to different extents and this may reflect characteristic affinities of particular nucleosides for the 'carrier'. Since none of the sugars or the purine and pyrimidine bases tested accelerated the exit of uridine, it is concluded that permeation of these compounds is mediated by separate 'carrier' systems.

The conclusion that the transport of nucleosides across the erythrocyte cell membrane is mediated by a specific facilitated diffusion system is supported by the

data of Kessel and Shurin (45) on nucleoside transport in cells of the L1210/CA murine leukemia, and of Jacquez (44) on nucleoside transport in cells of the Ehrlich ascites carcinoma. However, some of the conclusions drawn in these and other previous investigations of nucleoside transport (Introduction, III (ii)) are not consistent with the results of the present study.

Kessel and Shurin observed that uranyl nitrate, a non-specific inhibitor of several membrane transport processes (70), caused an accumulation of nucleosides in L1210/CA cells; from this, they suggested that uranyl ion preferentially inhibits the efflux of nucleosides over the influx. This hypothesis requires further examination, since accumulative uptake by a transport system which appears to be independent of energy metabolism is thermodynamically unlikely. In the current study, the demonstration of counterflow of nucleosides, and the observation that NBzTGR inhibits both the influx and efflux of nucleosides in erythrocytes suggests that, at least in these cells, the same 'carrier' mediates both entry and exit; it is difficult to visualize a unidirectional inhibition of such a transport system.

Jacquez has proposed that nucleosides may enter cells of the Ehrlich ascites carcinoma by way of the glucose 'carrier' system. This suggestion was based on an observed 5 to 10 per cent inhibition of the uptake of 2 m*M* uridine in the presence of 20 m*M* galactose or 3-*O*-methylglucose; however, a considerably greater inhibition would be predicted if the

sugars were competing substrates. In the present study with erythrocytes, 2-deoxy-D-glucose and D-ribose (sugars with very high and very low affinities, respectively, for the glucose transport system (14)) did not promote the counterflow of uridine, NBzTGR did not block glucose uptake, and the rate of uptake of 2.5 m_M_ uridine was unaffected by the presence of 2.5 m_M_ 2-deoxy-D-glucose. These facts indicate that, at least in human erythrocytes, the facilitated diffusion system for nucleosides is distinct from that involved in the movement of sugars.

Scholtissek (50) has suggested that the transport of nucleosides in cultured chick fibroblasts may involve formation of a complex between the transport substrates and the particular kinases responsible for their phosphorylation. This theory proposes that a nucleoside which is not phosphorylated will not be transported, and it predicts, for instance, that the transport site for uridine and cytidine will be different from that for adenosine, because the former nucleosides are substrates of a separate kinase. These ideas are not compatible with the present study which showed that uridine is not phosphorylated in human erythrocytes and yet shares a common transport system with cytidine, adenosine and several other nucleosides. As well, Kessel and Shurin have demonstrated mediated transport of cytosine arabinoside and deoxycytidine in tumor cells unable to phosphorylate either nucleoside; these observations are not in agreement with the proposal of Scholtissek.

In _E_. _coli_, a 'carrier' with broad specificity for

nucleosides has been recognised (51,52); this 'carrier' system has several properties which are similar to those of the facilitated diffusion system for nucleosides in human erythrocytes. However, the bacterial cells differ from erythrocytes in being capable of accumulative uptake, and in having a two-element transport system in which reaction with specific permeases is thought to precede transport by the 'carrier'.

The present study of the mechanism of action of NBzTGR supports other evidence from this laboratory that the nucleoside analogue is an irreversible, competitive inhibitor of nucleoside transport (Introduction, III (ii)). NBzTGR reacted with the transport system in a time- and temperature-dependent manner, to produce an apparently irreversible inhibition of nucleoside movement across the membranes of intact erythrocytes and of erythrocyte 'ghosts'; these results suggest that it may inactivate a membrane-bound 'carrier' which mediates the influx and efflux of nucleosides in these cells. High concentrations of uridine were able to partially protect the transport system against inactivation, indicating that NBzTGR may compete with nucleosides for a common binding site. The nucleoside analogue did not affect influx of purine and pyrimidine bases, of sugars or of leucine, and these facts imply that NBzTGR specifically inhibits the facilitated diffusion of nucleosides. Preincubation of a 30% cell suspension for 5 minutes with $7 \times 10^{-7}\underline{M}$ NBzTGR reduced the rate of uridine uptake into these cells by 70% (Figure 7). This potent effect at low concentrations suggests that the inhibitor

may bind specifically with the nucleoside transport system; however, more data are required to confirm this hypothesis.

The mechanism of the inhibitor-erythrocyte interaction is not yet understood. Extensive washing of inhibitor-treated cells with medium which contains nucleosides may show if the effect of NBzTGR on the transport system is irreversible, or if it involves a pseudo-irreversible reaction in which the affinity of the inhibitor for the 'carrier' is sufficiently high to give the appearance of irreversibility (66). Comparison of the extents to which ring- and benzyl-labelled inhibitor molecules are able to bind to 'ghosts' may indicate if alkylation is involved in the reaction of inhibitor with the cell membrane; measurement of the amount of radioactivity bound, at saturation, to a known number of cells may also allow calculation of the number of transport sites per cell, providing non-specific binding of the inhibitor can be excluded.

In the current investigation it was found that BzTGR, but not BzTG, blocked uridine uptake, in human erythrocytes. This is consistent with previous evidence from this laboratory that there is considerable structural specificity, particularly at positions 6 and 9, for inhibition of nucleoside metabolism by derivatives of 6-alkylmercaptopurine ribonucleosides (41,42). Extended structure-activity studies may yield further information about the mechanism by which 6-alkylmercaptopurine ribonucleosides bind with the 'carrier' system and inhibit nucleoside transport.

It appears quite possible that the apparently

irreversible and competitive inhibition of nucleoside transport produced by NBzTGR may be useful in isolating and characterising the facilitated diffusion system for nucleosides. A number of techniques which may be employed in this study are suggested by previous attempts to isolate other transport systems.

In bacteria, several relatively simple approaches to the isolation of transport systems are available (71). For example, several bacterial transport systems are inducible, and proteins have been isolated from induced cells which cannot be detected in non-induced cells or in mutants lacking a particular permease; proteins thought to be involved in the transport of β-galactosides in *E. coli* have been identified in this manner. An osmotic shock technique has also been used to isolate components of bacterial transport systems. Osmotic shock produces an impairment of uptake of several compounds, accompanied by appearance in the shock fluid of proteins capable of binding to specific permeants; the transport processes can be partially restored during incubation of the treated cells with these proteins.

Unfortunately, these techniques are not applicable to mammalian cells in which transport systems are not thought to be inducible, and osmotic shock is not known to liberate proteins involved in transport. A number of alternative methods have been used in attempts to isolate the erythrocyte sugar transport system.

LeFevre *et al*. (72) and Mawdesley and Widdas (73) have found that specific lipids from the erythrocyte membrane can

form complexes with sugars. These complexes are stable in organic solvents but dissociate in water, and their formation is inhibited by dinitrofluorobenzene (DNFB), an inhibitor of sugar transport in erythrocytes. However, in forming these complexes, the lipids do not show the optical specificity characteristic of the intact transport system and so it is doubtful if they are involved in the movement of sugars across the erythrocyte membrane.

A protein which binds glucose has been detected by Langdon and Sloan (74). They postulated that glucose transport might involve transient formation of a Schiff's base between the aldehyde group of the sugar and a lysyl side-chain of the 'carrier' protein; this could be reduced chemically to form a stable secondary amine as shown:

$$R'.CHO + H_2NCH_2.R^2 \rightleftarrows R'.CH = NCH_2.R^2 \xrightarrow{H_2} R'.CH_2NHCH_2.R^2$$

Langdon and Sloan found experimentally that incubation of either erythrocyte 'ghosts' or membrane proteins with radioactive glucose, in the presence of sodium borohydride, resulted in incorporation of label in a stable form, and they identified the glucose-accepting site as a lysine residue.

Stein and coworkers have used the structural and stereochemical specificity of sugar binding to monitor attempts to isolate the erythrocyte sugar transport system. They have developed a retardation technique (75-77) to detect the presence of the glucose 'carrier' in fragments of the erythrocyte membrane. In this procedure, membrane fragments and an inert material, such as 'Celite' or DEAE-cellulose,

is packed into a column, a solution of two sugars, usually D-glucose-^{3}H and sorbose-^{14}C or L-glucose-^{14}C, is applied, and the compounds are eluted from the column. If the transport system is present, specific binding of D-glucose to the 'carrier' results in a partial separation of the sugars; the extent of separation depends on the number of available binding sites, since binding with the transport system is a saturable process, and on the dissociation constant of the glucose-'carrier' complex. The differential effect is eliminated by treating the columns with $10^{-4}\underline{M}$ phloretin, an inhibitor of sugar transport in intact cells, or by incubating erythrocytes with DNFB before incorporation of the membrane proteins of these cells into the column.

This group has also reported an ultra-filtration technique (77), in which a solubilised and concentrated extract of cell membrane is added to a solution of L-glucose-^{14}C and D-glucose-^{3}H, and the mixture is placed in a dialysis bag, and the dialysate is collected and analysed for radioactivity. If the material within the bag is able to bind D-glucose, the emerging radioactivity is poor in tritium when compared with a control solution dialysed in the absence of membrane extract. They have fractionated membrane proteins by salt-fractionation and ion-exchange chromatography, and, by use of the ultra-filtration technique to detect proteins capable of retarding the efflux of glucose, have obtained a glucose-binding protein with a molecular weight of approximately 45,000 (78).

Similar techniques may allow isolation from the erythrocyte membrane of a protein fraction which binds reversibly with nucleosides and irreversibly with NBzTGR. If it can be established that the facilitated diffusion system is specifically and irreversibly labelled with radioactive NBzTGR, it should be possible to 'tag' the transport system with this compound; solubilization and fractionation of the membrane proteins would then permit isolation of a radioactive NBzTGR-'carrier' complex. This study would be analogous to that of Langdon and Sloan, who were able to irreversibly label a protein thought to be involved in erythrocyte sugar transport; a similar study by Hokin _et_ _al_. (79), involved the use of strophanthidin-3-haloacetates to specifically and irreversibly label the cardiotonic steroid site of the brain transport ATPase.

Ultrafiltration and retardation studies may also permit isolation of the nucleoside 'carrier', and NBzTGR may be of value in assessing these investigations. For instance, if addition of the inhibitor to a particular protein fraction eliminates differential binding effects between a pair of nucleosides with high and low affinities, respectively, for the facilitated diffusion system, this will indicate that such effects are due to the presence of the nucleoside 'carrier' in the fraction and not to a difference in the abilities of the added compounds to bind non-specifically with other membrane proteins.

The inhibitory properties of NBzTGR and related 6-alkylmercaptopurine ribonucleosides appear to be analogous to those of 'active-site-directed' irreversible enzyme inhibitors (80,81); the latter compounds bind in a specific covalent manner to the active sites of certain enzymes, and have been used extensively in investigations of the mechanism of enzyme action. It seems possible that kinetic studies of effects of transport inhibitors on the isolated transport system may yield information about the mechanism of binding of substrates to the nucleoside 'carrier', and may also allow examination of the conformational changes in the 'carrier' which have been postulated to accompany the binding of permeants with mediated transport systems.

APPENDIX

I. The equilibrium distribution of uridine

The experimental procedure has been described in the text, and the results are tabulated below. Numbers in parentheses represent the number of individual determinations of each value; the mean deviations of these values are given.

(a) Total water in the packed cells from suspension (a)

(i) Wet weight/ml of cells = 1.149 ± 0.002 g (5)

(ii) Dry weight/ml of cells = 0.275 ± 0.002 g (5)

= 23.9% of wet weight/ml of cells

Therefore, 1.0 ml of packed cells contains 76.1% water.

(b) Extracellular water in the packed cells from suspension (b)

(i) Inulin-carboxyl-^{14}C/10 µl of medium = 13,436 ± 24 cpm (5)

Inulin-carboxyl-^{14}C/ml of medium = 1.34×10^6 cpm

(ii) Inulin-carboxyl-^{14}C/0.5 ml of neutralised cell extract = 17,480 ± 243 cpm (10)

Inulin-carboxyl-^{14}C/ml of cells = 1.05×10^5 cpm

= 7.8% of cpm/ml of medium

Therefore, 1.0 ml of packed cells contains 7.8% extracellular water.

(c) Distribution of uridine between the medium and intracellular water of suspension (c)

Specific activity of uridine-2-^{14}C = 2.08 x 10^{5} cpm/μmole

(i) Uridine-2-^{14}C/50 μl of medium = 10,016 ± 68 cpm (5)

Therefore, uridine in the medium = 0.96 mM

(ii) Uridine-2-^{14}C/0.2 ml of neutralised cell extract = 11,083 ± 273 cpm (10)

Uridine/ml of packed cells = 0.80 μmoles

From (a) and (b), 1.0 ml of packed cells contains 0.08 ml of extracellular water and 0.68 ml of intracellular water.

Uridine/0.68 ml of intracellular water = 0.72 μmoles

Therefore, uridine in the intracellular water = 1.06 mM

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